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Genetic variation is associated with *RTN4R* expression and working memory processing in healthy humans

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HIGHLIGHTS:

- rs696884 impacts gene expression and physiology of prefrontal cortex.
- rs696884 AA genotype is associated with lower *RTN4R* mRNA expression
- rs696884 modulates dorsolateral prefrontal activity during working memory processing.

ABSTRACT

The Nogo receptor (NgR) is implicated in neurodevelopmental processes and it participates in inhibiting axonal growth. Consistent with its high levels of expression in the prefrontal cortex, animal studies indicate that NgR is relevant for prefrontal-related cognitive processing. Given that genetic variation may alter mechanisms of gene expression impacting molecular and systems-level phenotypes, we investigated the association of genetic variation with the expression of the NgR coding gene (*RTN4R*), as well as with prefrontal correlates at progressively greater biological distance from gene effects. First, we studied the association of single nucleotide polymorphisms (SNPs) with *RTN4R* mRNA expression in postmortem prefrontal cortex of humans without psychiatric illnesses. Then, we probed in peripheral blood mononuclear cells (PBMCs) the association that we found in prefrontal tissue. Thus, we investigated whether functional genetic variation affecting *RTN4R* expression is also associated with prefrontal activity during working memory. We found that rs696884 (A/G) predicted these phenotypes. Specifically, the AA genotype was associated with lower *RTN4R* mRNA expression levels in the prefrontal cortex and PBMCs and inefficient prefrontal activity during working memory compared to the GG genotype. These results suggest that genetic variation associated with *RTN4R* mRNA expression influences prefrontal physiology in healthy individuals. Furthermore, they highlight the need for further investigations of the role of NgR in the pathophysiology of brain disorders associated with prefrontal dysfunction.

KEYWORDS: rs696884, Nogo Receptor, mRNA expression; prefrontal activity, working memory

1. INTRODUCTION

Mechanisms regulating neuronal plasticity participate in the maturation of the brain and are critical determinants of brain function, including cognition (Hoistad *et al.*, 2009). The Nogo receptor (NgR) complex has been implicated in the refinement of neuronal connectivity and it plays

a crucial role in limiting neuronal regeneration, sprouting, and plasticity (Akbik *et al.*, 2012). Three myelin-associated growth inhibitory factors including Nogo-A, myelin associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp), share NgR as a receptor. These factors regulate cytoskeletal dynamics and inhibit axonal growth (Atwal *et al.*, 2008, Chen *et al.*, 2000, Fournier *et al.*, 2001, Lai *et al.*, 1987, Wang *et al.*, 2002).

NgR is a glycosylphosphatidylinositol- (GPI-) anchored protein without a transmembrane domain (Barton *et al.*, 2003, Fournier *et al.*, 2001), it is highly expressed in the frontal cortex of animals and humans (Josephson *et al.*, 2002; http://www.gtexportal.org), and it is localized to axonal membranes pre- and post-synaptically (Wang X. *et al.*, 2002). Its signaling transduction requires membrane-associated co-receptors (Mi *et al.*, 2004), allowing it to inhibit neurite outgrowth via the RhoA/ROCK/LIMK1/Rac1 pathway (Fournier *et al.*, 2003, Hsieh *et al.*, 2006, Mi *et al.*, 2004, Niederost *et al.*, 2002). Accordingly, genetic deletion or pharmacological inhibition of NgR enhance axonal plasticity after injury (Hanell *et al.*, 2010, Lee *et al.*, 2004, Mingorance *et al.*, 2004, Zheng *et al.*, 2005).

Other genetic evidence in animal models suggests that NgR signaling is also involved in cognitive processing, including those processes mediated by the prefrontal cortex (Budel *et al.*, 2008, Hsu *et al.*, 2007, Karlen *et al.*, 2009, Van Gaalen *et al.*, 2012, Willi *et al.*, 2010). For example, NgR knock-out mice have impaired spatial working memory and slower acquisition of spatial learning and memory (Budel *et al.*, 2008, Van Gaalen *et al.*, 2012). These findings are consistent with genetic evidence indicating that a hemizygous microdeletion occurring at the NgR gene locus (*RTN4R* - 22q11.2) is associated with prefrontal-related cognitive impairments in humans, including working memory, conflict monitoring, and visuo-spatial short-term memory deficits (Antshel *et al.*, 2005, Bearden *et al.*, 2001, Sobin *et al.*, 2005, Woodin *et al.*, 2001).

A series of studies also indicate that variations within *RTN4R* may have functional consequences for phenotypes at the molecular and behavioral level. In this regard, four *RTN4R* missense mutations have been associated with lower NogoA binding to NgR and lower inhibition of

NogoA-NgR mediated axonal growth (Budel *et al.*, 2008). Another missense mutation predicted mild impairment of spatial memory performance in mice (Lazar *et al.*, 2011). Moreover, the relevance of the variations in this gene to the development of brain disorders has been suggested by other studies that implicated several rare coding variants and single nucleotide polymorphisms (SNPs) in the *RTN4R* gene in the pathogenesis of schizophrenia (Budel *et al.*, 2008, Hsu *et al.*, 2007, Liu *et al.*, 2002, Sinibaldi *et al.*, 2004). Consistently, previous evidence has suggested that the *RTN4R* genetic region (22q11) is relevant to this brain disorder (Pulver *et al.* 1994; Murphy *et al.* 1999; Karayiorgou *et al.* 1995).

The aim of this study was to investigate the association of *RTN4R* genetic variations with prefrontal molecular and imaging phenotypes in healthy humans. Given the widespread expression of NgR in the prefrontal cortex (Josephson *et al.*, 2002), we focused on association of *RTN4R* variation with gene expression in this brain region. Furthermore, given the relevance of NgR for prefrontal-related cognitive processing (Karlsson *et al.*, 2013, Lazar *et al.*, 2011, Tong *et al.*, 2013), we also investigated the association of functional *RTN4R* variation with prefrontal activity during working memory processing, which is tightly linked with this brain region.

We used a hierarchical and stepwise translational genetic approach, which included both postmortem and *in vivo* experiments. In particular, we first identified *RTN4R* variation predicting *RTN4R* mRNA expression levels in postmortem human prefrontal cortex samples. Second, in order to extend these results to another tissue, we investigated the association between *RTN4R* variation and mRNA expression in peripheral blood mononuclear cells (PBMCs). Then, we also tested whether *RTN4R* functional genetic variation modulates prefrontal activity during working memory processing. We hypothesized that there was an association between *RTN4R* genetic variation and the molecular and imaging phenotypes that we investigated.

2. MATERIAL AND METHODS

2.1 Postmortem study

2.1.2 Association of *RTN4R* SNPs with prefrontal *RTN4R* mRNA expression levels

Expression and genotype data from prefrontal tissues of 258 Caucasian (N = 112) and African American (N = 146) humans without psychiatric illness (90 females; mean [SD] age at death: 27.4 [22] years; RNA Integrity Number (RIN): 8.4 [0.9]; *postmortem* interval (PMI): 26.0 [17.3] hours; PH: 6.5 [0.3]) were obtained using Braincloud, a publicly available database (www.braincloud.jhmi.edu) (Colantuoni *et al.*, 2011). Subjects with evidence of macro- or microscopic neuropathology, drug or alcohol abuse, or psychiatric illness were excluded. RNA from prefrontal grey matter for each subject was analyzed using spotted oligonucleotide microarrays, yielding data from 30,176 gene expression probes. In the present study, we focused on *RTN4R* mRNA expression, which included two probes in this dataset (Clone Index 23242 and 15361). In order to investigate the relationship between race and *RTN4R* expression, we performed an ANCOVA with *RTN4R* postmortem prefrontal expression as the dependent variable, race as the independent variable, and age at death, gender, RIN, PMI, and PH as nuisance covariates. Even if we found an effect of race on *RTN4R* mRNA expression levels, we still investigated both Caucasians and African Americans together to increase the sample size.

DNA from cerebellar tissue was characterized in Braincloud with Illumina BeadChips, producing 625,439 SNP genotypes for each subject (Colantuoni *et al.*, 2011). We examined 41 of these SNPs, which spanned 100 Kb upstream and downstream of *RTN4R*. Thus, separate ANCOVAs were performed to investigate the association of these SNPs with *RTN4R* expression as measured with each of the two probes, using age at death, PMI, PH, RIN, and gender as covariates of no interest. Results underwent Bonferroni corrections at $p < 0.05$ for the number of *RTN4R* SNPs investigated (N = 41) and the number of probes (N = 2).

Once we identified the SNP associated with *RTN4R* expression levels, we performed separate ANCOVAs in African Americans (N= 93 AA, 51 AG, 2 GG) and Caucasians (N= 25 AA, 67 AG, 20 GG), given the association between expression of *RTN4R* and race (see results). Here, *RTN4R* gene expression was the dependent variable, the genotype of interest (rs696884, see results)

was the independent variable, and age, gender, RIN, PMI, and PH were the nuisance covariates. Since the GG group in African Americans included only two subjects, we collapsed AG and GG individuals together in a G carrier group (93 AA, 53 G carrier) while investigating genotype expression patterns in this race.

2.2 *In vivo* study

2.2.1 Subjects and genotyping

The experimental protocol was approved by the local institutional review board and carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Participants gave written informed consent before being enrolled in the study. Overall, 173 healthy Caucasian subjects from the region of Apulia, Italy participated in the study. Exclusion criteria were the presence of a neurological or psychiatric disorder, a first-degree relative affected by a psychiatric disease, brain trauma with loss of consciousness, alcohol or drug abuse, and an Intelligence Quotient (IQ) lower than 80 measured with the Wechsler Adult Intelligence Scale-Revised (WAIS-R). All subjects underwent structured clinical interviews according to the DSM-IV (SCID-I non-patient's edition) to exclude any psychiatric disorders. Furthermore, the experimental protocol included blood sampling for DNA extraction and a functional magnetic resonance imaging (fMRI) session.

DNA was extracted from the peripheral blood of all individuals using standard procedures (Kiagen Midi Kit). Genotyping was then performed using the Illumina Infinium HD Gemini 1M Duo BeadChips platform, which allowed the generation of genome-wide data. For the purpose of this study, we focused on the *RTN4R* variation (rs696884) selected with the postmortem investigation. ANOVA and χ^2 tests were used to compare demographics (age, gender, socio-economic status), handedness, and IQ as functions of rs696884.

2.3 Association of rs696884 with *RTN4R* expression in peripheral blood mononuclear cells (PBMCs)

The association between *RTN4R* genetic variations (rs696884) and mRNA expression levels found in postmortem prefrontal tissues was also investigated *in vivo* in PBMCs from 41 healthy Caucasian individuals (AA = 14, AG = 16, GG = 11). Genotype groups were in Hardy-Weinberg Equilibrium (χ^2 = 1.9; p = 0.16) and matched in terms of age and gender (all p > 0.2). The mRNA expressions levels were analyzed by quantitative real-time polymerase chain reaction (qPCR). In particular, peripheral blood was collected in Tempus™ Blood tubes (Life Technologies, Carlsbad, CA, USA), and all samples were frozen and stored at −20°C. RNA was extracted with the Tempus™ Spin RNA Isolation Kit (Life Technologies) and stored at −80°C. RNA concentrations and purity were evaluated with an Epoch spectrophotometer (Biotek, Winooski, VT, USA). Reverse-transcription (RT) of the total RNA into single-stranded cDNA was performed using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). *RTN4R* expression was assessed using a TaqMan® probe (assay ID qHsaCEP0024213) from Bio-Rad (Hercules, CA, USA). The levels of *RTN4R* were normalized to *actin B* (*ACTB*) (4333762F, Life Technologies). Next, qPCR (20 µL reaction) was performed under standard conditions on an MJ Mini Opticon instrument (Bio-Rad) in triplicate. A negative control and a calibrator sample consisting of cDNA pooled from all samples were included for each assay.

The relative expression of *RTN4R* was determined by performing the comparative method. First, data were normalized to *ACTB* and then to the calibrator. The results were expressed as $2^{-\Delta\Delta Ct}$, where ΔCt of each sample was defined as Ct (target gene = *RTN4R*) – Ct (reference gene = *ACTB*), and ΔΔCt = ΔCt sample - ΔCt calibrator. Finally, ANOVA was performed to evaluate the association between the genotypes and mRNA expression levels.

2.4 Association of rs696884 with prefrontal activity during working memory processing

2.4.1 Subjects and task

159 subjects underwent functional magnetic resonance imaging (fMRI) while performing the N-Back working memory task (Callicott *et al.*, 1999). Rs696884 genotype groups (AA = 44, AG = 74, GG = 41) were matched with respect to age, socio-economic status, handedness, and IQ (all $p > 0.05$), but not for gender ($\chi^2 = 6.5$, $p = 0.03$). The genotype distribution was in Hardy-Weinberg Equilibrium ($\chi^2 = 0.75$, $p = 0.3$). "N-back" referred to how far back in the sequence of stimuli the participant could recall. Stimuli consisted of numbers (from 1 to 4) shown randomly and displayed at the points of a diamond-shaped box. During the control condition (0-back) subjects were required to press on the button box the same stimuli displayed on the screen. During the memory condition (2-back), subjects had to recall the number shown for two stimuli before the one currently seen, while continuing to encode the stimuli as they appeared on the screen. Eight control and memory conditions of 30 seconds each alternated in a block design for a total of 4 minutes and 8 seconds. Stimuli were presented via a back-projection system. Responses were recorded through a fiber optic button box allowing measurements of accuracy (percent correct responses) and reaction times (milliseconds).

2.4.2 fMRI data acquisition and analysis

The fMRI data were acquired on a General Electric 3 Tesla scanner using gradient-eco-planar-imaging sequences (20 contiguous slices; TR/TE = 2000/30 ms; field of view: 24 cm; matrix: 64 × 64). Image analyses were performed using SPM8. Images were realigned, corrected for head motion, normalized into the MNI space, and smoothed with a 10 mm Gaussian filter. The data set included individuals with head movement < 2 mm of translation and < 1.5 mm of rotation. Residual movement was used as a regressor of no interest. Individual contrast images of 2-back versus 0-back were used in a random-effects model for group level analyses. An ANCOVA was performed with genotype as the independent variable and gender as the covariate of no interest. Results were constrained by a mask obtained by combining group activation maps for all participants. We used a statistical threshold of $p < 0.05$. This p-value was family-wise corrected

within our region of interest, which was the prefrontal cortex. More specifically, this correction was performed within BA 46 (as identified with the Wake Forest University PickAtlas - http://fmri.wfubmc.edu/cms/software#PickAtlas), which was the prefrontal region where the cluster of interest was located. We adopted this procedure to reduce the number of multiple comparisons. Blood-oxygen- level-dependent (BOLD) parameter estimates were extracted from the significant cluster using MarsBar and investigated with a post hoc Fisher's test. Pearson's test was used to determine the relationship between behavior and brain activity. ANCOVA with gender as a covariate of no interest was also performed on behavioral data acquired during scan acquisition.

3. RESULTS

3.1 Postmortem investigation

3.1.2 Association between rs696884 and *RTN4R* mRNA expression levels in the postmortem prefrontal cortex

ANCOVA indicated that Caucasian subjects had higher *RTN4R* expression levels than African Americans ($F = 17.79$, $p = 0.00003$). Separate ANCOVAs indicated an association between rs696884 (A/G) and *RTN4R* mRNA expression levels in the postmortem prefrontal cortex ($F = 8.3$; $DF = 2$; $p = 0.02$ after Bonferroni correction). Fisher's post hoc analysis revealed that AA subjects had lower *RTN4R* mRNA expression levels relative to GG ($p = 0.00003$) and AG ($p = 0.02$) individuals. Furthermore, GG subjects had higher mRNA expression levels compared with AG individuals ($p = 0.003$) (Figure 1a). No other significant SNPs/mRNA expression associations were found.

Post hoc ANCOVA in the subsample of Caucasian individuals indicated that there was a main effect of the rs696884 genotype on *RTN4R* expression ($F = 3.07$; $DF = 2$; $p = 0.05$). Fisher post hoc analysis revealed lower mRNA expression levels in AA and AG compared to GG individuals ($p = 0.03$). No differences were present between the AA and AG groups ($p = 0.1$). Finally, ANCOVA in African Americans did not indicate an effect of genotype on gene expression

(F = 1.3, p = 0.2). However, the average expression in AA subjects was lower than that in G carriers (A carrier = –0.30 and G carrier = –0.17), which was consistent with the association of the genotype in the overall group and in Caucasians.

3.2 *In vivo* investigation

3.2.1 Association of rs696884 with *RTN4R* mRNA expression levels in PBMCs

Consistent with the postmortem findings, ANOVA indicated that there was an effect of rs696884 on *RTN4R* mRNA expression levels in PBMCs of healthy Caucasian individuals (F = 5.7; DF = 2; p = 0.006). Fisher's post hoc test revealed that AA individuals had lower mRNA expression levels compared to GG (p = 0.001) and AG subjects (p = 0.05). Meanwhile, no differences were found between AG and GG subjects (p = 0.1) (Figure 1b).

3.2.2 Association between rs696884 and prefrontal activity during working memory

There was no effect of genotype on behavioral data (accuracy p = 0.4; reaction time p = 0.8) in the fMRI samples. Thus, the genotype effect that we found on prefrontal activity reflected how the brain processed working memory, and not how individuals scored on the task. SPM8 ANCOVA revealed that there was an effect of the genotype on the left dorsolateral prefrontal cortex (BA46; x = –34 y = 34 z = 16; K = 54; Z = 3.4; FWE p = 0.05). Fisher's post hoc test on BOLD values extracted from this significant cluster indicated that AA subjects had higher levels of activity compared to either AG (p = 0.001) or GG individuals (p = 0.001). No significant differences were present between the GG and AG genotype groups (p = 0.6) (Figure 2a–b).

Pearson's test indicated a negative correlation between parameter estimates extracted from the cluster associated with the main effect of the genotype and behavioral accuracy in AA individuals (r = –0.3, p = 0.01). This correlation was not present in AG (r = –0.1, p = 0.2) or GG (r = –0.09, p = 0.5) subjects (Figure 3a–c).

4. DISCUSSION

The results of the present study indicate an association between *RTN4R* genetic variation and phenotypes linked to prefrontal function. In our hierarchical approach, we selected a SNP (rs696884) based on its association with *RTN4R* postmortem mRNA expression in the prefrontal cortex. Then, we extended this finding in PBMCs of healthy humans. Furthermore, we found that this SNP also modulated prefrontal activity during working memory processing.

Rs696884 is an intergenic SNP located downstream of the *RTN4R* locus at position 20183509 (GRCh37.p13). This polymorphism has not been investigated previously. We found that the AA genotype of this SNP was associated with lower *RTN4R* mRNA expression levels in the postmortem prefrontal cortex compared to the AG and GG genotypes. Additionally, we obtained similar results for the PBMCs of healthy subjects. Thus, our findings suggest that rs696884 may modulate the expression of *RTN4R* and possibly affect NgR function and signaling.

In our study, rs696884 also influenced prefrontal function during working memory processing. In particular, we found that subjects with the AA genotype had greater dorsolateral prefrontal cortex activity during the 2-back condition compared to individuals with the G allele. This occurred despite the lack of genotype effects on behavioral performance. One interpretation of this finding is that AA individuals may need to recruit more neuronal resources to perform the task compared to AG and GG subjects, even though they displayed similar behavioral performance. In other words, greater prefrontal activity in our study may be an indicator of less efficient working memory processing in the prefrontal cortex of AA individuals, which has also been suggested by previous studies (Blasi *et al.*, 2013, Callicott *et al.*, 2003). Consistent with this interpretation, we found a negative correlation between behavioral accuracy and prefrontal activity during the 2-back condition in AA subjects. Thus, greater prefrontal engagement predicted lower behavioral proficiency in these individuals. Interestingly, this relationship was not observed for the other genotypic groups. This finding suggests that the functional role of the significant prefrontal cluster is less relevant for working memory processing in AG and GG individuals.

Rs696884 is an intergenic SNP located downstream of *RTN4R*. The molecular link between this polymorphism and expression of the *RTN4R* gene has yet to be elucidated. The UCSC genome browser (https://genome.ucsc.edu/) indicates that the DNA sequence that includes rs696884 is bound by the transcription factor CTCF. This is a zinc-finger protein involved in transcriptional regulation (Ohlsson *et al.*, 2001) and it acts as an insulator that blocks enhancer activity (Bell *et al.*, 1999, Hou *et al.*, 2008). A previous study indicated that constitutive CTCF loci seem to rely distant from transcription starting sites (Li *et al.*, 2013) and one of the CTCF binding sites near the rs696884 locus has been identified (Li *et al.*, 2013). Thus, it is possible that a mechanism by which rs696884 affects *RTN4R* expression relies on CTCF. However, further studies should address this topic.

Some limitations of the study must be acknowledged. First, in the postmortem study, we found an association between race and *RTN4R* expression. On the other hand, the separate analysis of African Americans and Caucasians suggested that the two races have a consistent directionality in the relationship between rs696884 and *RTN4R*. The lower sample size may decrease power of detection of *RTN4R* effects. Thus, it is possible that population stratification might have biased the results. Another limitation is that the sample size for the PBMCs study was small. However, the consistency between the PBMC and postmortem findings may suggest that the association between genotype and mRNA expression in the peripheral blood is not driven by type I errors. An additional limitation involved the imaging study. Here, we reduced the number of multiple comparisons for statistical correction procedures using a sub-area within our region of interest (i.e., the prefrontal cortex). Indeed, this brain region is a very large area with multifaceted structural and functional characteristics. This may biologically justify our statistical approach. Furthermore, the consistencies between the molecular and fMRI results suggest that the findings that we report are not artefactual. Further studies with larger sample sizes might help to increase the power of detection of subtle genetic effects of *RTN4R* on complex phenotypes.

In conclusion, the hierarchical model used in this study indicates an association between a genetic variation affecting *RTN4R* expression and molecular and imaging phenotypes related to the prefrontal cortex. These findings suggest that a genetic predisposition for lower *RTN4R* mRNA expression levels may confer risks for sub-optimal patterns of prefrontal phenotypes relevant for complex brain disorders.

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LLB, GB, and AB designed the experiments; MTA, ST, RM, and AR equally contributed to the acquisition, analysis, and interpretation of the genetic data; ED, ADG, LAF, LEF, and BG equally contributed to the acquisition, analysis, and interpretation of imaging and behavioral data; LLB, GB, and AB wrote the paper; GB and AB revised the paper; all the authors gave their final approval of the version to be published.

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CONFLICTS OF INTEREST

There are no known conflicts of interest associated with this publication.

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FIGURE CAPTIONS

Figure 1.

Association between rs696884 and *RTN4R* mRNA expression levels in postmortem prefrontal cortex and PBMc. a) Scatterplot of *RTN4R* expression in postmortem prefrontal cortex as a function of rs696884 in Caucasians and African Americans. b) Main effect of rs696884 on *RTN4R* mRNA expression levels in PBMC. AA individuals were associated with lower expression compared to AG and GG subjects. The mRNA expression was measured using the $2^{-\Delta\Delta Ct}$ method. Error bars indicate a 95% confidence interval. See text for statistics.

Figure 2.

Association between rs696884 and prefrontal activity during working memory processing. a) Rendering of the brain showing the dorsolateral prefrontal functional cluster associated with a main effect of rs696884 during the 2-back working memory task (BA46, x = –34 y = 34 z = 16). b) Parameter estimates extracted from the cluster displayed in a). AA subjects had greater dorsolateral prefrontal activity compared to AG and GG individuals. Error bars indicate a 95% confidence interval.

Figure 3.

Correlation between rs696884 and accuracy during working memory. Negative correlation between BOLD signal extracted from the cluster associated with a main effect of rs696884 during working memory and accuracy (percent correct responses) during the 2-back task in AA (a), but not AG (b) or GG (c), individuals. Dashed lines indicate a 95% confidence interval.

Fig. 1a

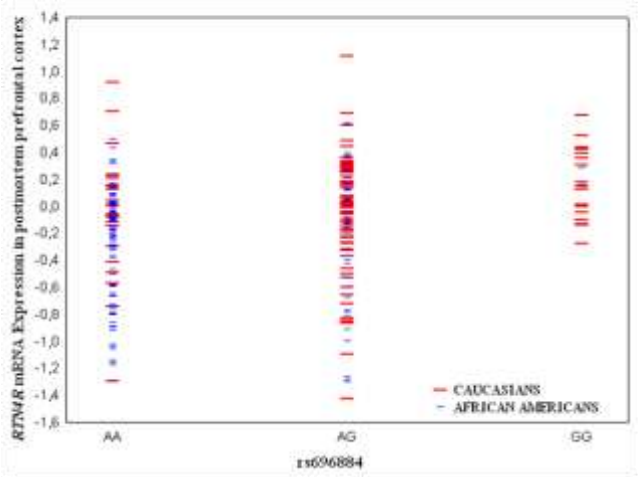

Fig. 1b

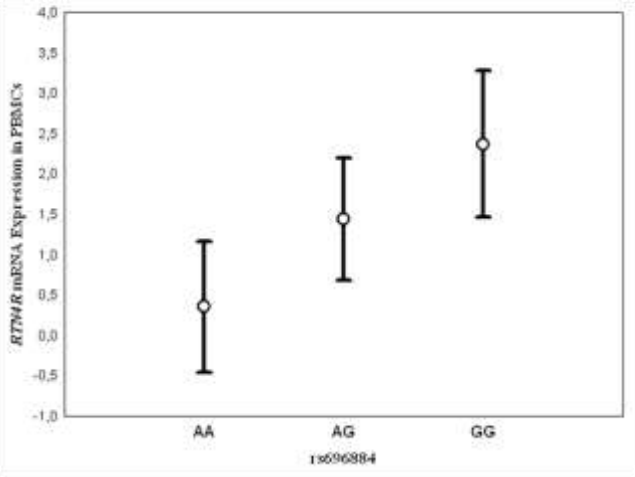

Fig. 2a

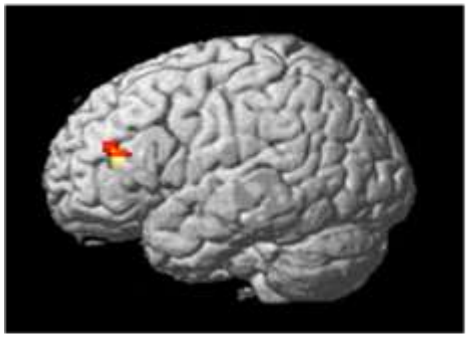

Fig. 2b

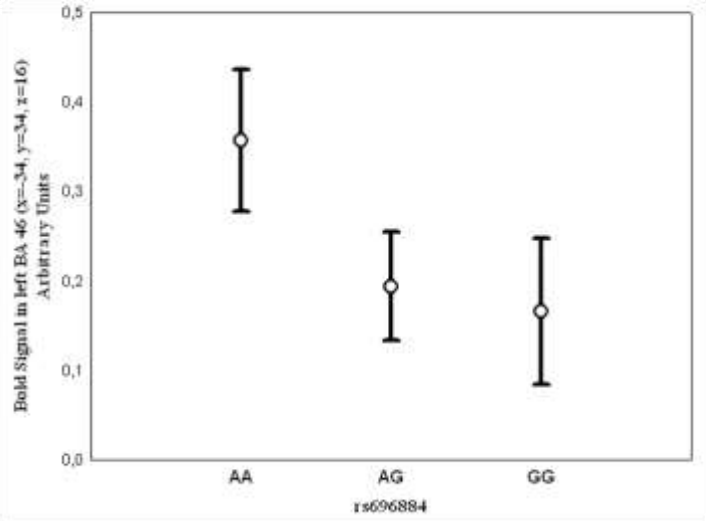

Fig. 3a

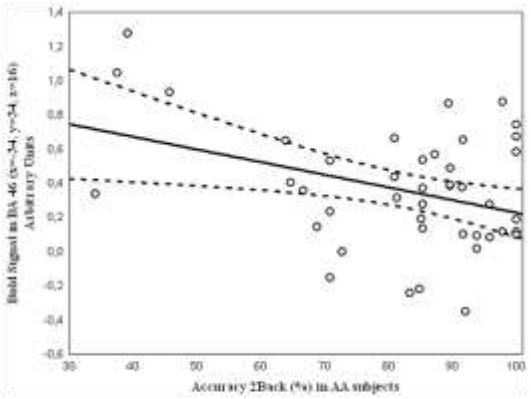

Fig. 3b

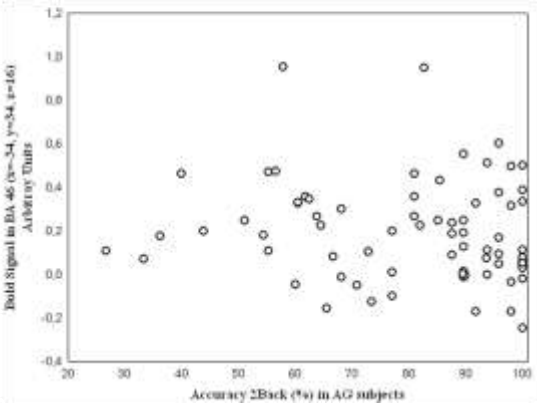

Fig. 3c